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Improved Androgenesis of Broccoli (*Brassica oleracea* var *italica*) Anthers Using Sucrose and Growth Regulators

[1,2]Magdi A.A. Mousa, [2]Ashraf G. Haridy, [2]Hassan S. Abbas and [2]Mohammed F. Mohammed

[1]Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia

[2]Department of Horticulture, Faculty of Agriculture, Assiut University, 71526, Assiut, Egypt

Corresponding Author: Magdi A.A. Mousa, Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia

ABSTRACT

The presented study was conducted to study the effects of BAP and its combinations with 2, 4-D and sucrose concentrations on androgenesis of broccoli cv. 'Arcadia'. The MS Basel media supplemented with AC (0.5 g L^{-1}) and 3% sucrose was used. The experiment was laid out in Completely Randomized Design (CRD) using 3 replicates. The effects of BAP in combination with 2, 4-D and sucrose concentrations on androgenesis of broccoli (cv Arcadia) anther cultures were investigated. The MS basal media supplemented with activated charcoal (AC) (0.5 g L^{-1}) was used. The medium complemented with BAP (1 mg L^{-1}) plus 2, 4-D (0.5 mg L^{-1}) significantly increased the percentage of anthers developing embryos (90%). Increasing 2, 4-D concentration (1 mg L^{-1}) significantly decreased the percentage of anthers formed embryos (10%). Regarding the regeneration of the formed embryos, the culture medium containing BAP (1 mg L^{-1})+2, 4-D (0.5 mg L^{-1}) or BAP (1 mg L^{-1})+2, 4-D (1 mg L^{-1}) produced the high percentages of embryos induced callus. The maximum percentage of embryos developed into plantlets were obtained with culture medium with BAP (1 mg L^{-1})+2, 4-D (0.5 mg L^{-1}) followed by BAP (1 mg L^{-1}) and 2, 4-D (1 mg L^{-1}). Concerning sucrose concentration, the culture medium with low sucrose concentration (20 g L^{-1}) enhanced the percentage of anthers developed embryos. The higher sucrose concentrations (50 and 60 g L^{-1}) increased percentages of dead anthers and anthers formed callus and decreased the percentage of embryos developing plantlets. BAP in combinations with 2, 4-D and low concentrations of sucrose can be successfully used to perform embryos and/or callus from the callus induction and embryos development either to callus or plants.

Key words: Broccoli androgenesis, BAP, another culture, sucrose, 2, 4-D

INTRODUCTION

Another culture is a useful plant tissue culture technique that can be successfully be used for production of homozygous lines. The production of double haploid lines using anther culture includes three steps: (a) Initiating androgenetic embryos, (b) Regenerating haploid plantlet from androgenetic embryos and (c) Doubling the chromosome number of the haploid plants (Lichter, 1982; Zhao *et al*., 1996; Palmer *et al*., 1996; Gorecka and Krzyzanowska, 2007). Anther and microspore cultures were used to produce double haploids in broccoli (Duijs *et al*., 1992;

Lee and Nam 1995; Yuan *et al*., 2011), Brussels sprouts (Biddington *et al*., 1993; Ockendon and McClenaghan, 1993) and cabbage (Higdon *et al*., 2007; Zhao *et al*., 2007; Yuan *et al*., 2011). Broccoli (*Brassica oleracea* var *italica*) (2n = 2x = 18) is widely consumed and grown vegetable crops worldwide. During the last decade, broccoli received a great attention of seed companies due to the increase production and its association with lower incidence of certain cancers including lung, colon, breast, ovarian and bladder cancers (Higdon *et al*., 2007; Zhao *et al*., 2007; Yuan *et al*., 2011). Broccoli is a cross-pollination crop and the production of high yield and quality broccoli homozygote/pure lines required intensive work and time. High genetically uniform lines/cultivars (pure line and homozygote line) with desired traits are urgently required before start plant breeding program. The response of broccoli anther culture varied based on crop genotypes, growth conditions of donor plants, microsporogensis developmental stage, growth medium and pretreatments of the flower buds (Higdon *et al*., 2007; Zhao *et al*., 2007; Yuan *et al*., 2011). Embryo production of broccoli anther culture could be improved via combined cold and heat shock pretreatments of the flower (Yuan *et al*., 2011) and addition of activated charcoal to the culture medium (Da Silva Dias, 1999). Heat shock treatments and NLN-13 medium was successful to enhance emberyogenesis of 6 subspecies of *Brassica oleracea* (Duijs *et al*., 1992). Da Silva Dias 1999 reported that the percentage of embryogenesis increased in broccoli by the addition of activated charcoal to the culture medium. The present study aimed to investigate the effects of BAP in combination with 2, 4-D and the media sucrose content on androgenesis of broccoli.

MATERIALS AND METHODS

Plant materials: The first formed flower buds of 2-3 mm were collected from the donor plants for anther culture. The donor plants belong to the broccoli cv. 'Arcadia' which is grown in autumn and winter seasons in Saudi Arabia. The stage of microsporogenesis was determined by microscopic observation of crushed specimens in hematoxyline (Fig. 2a-b).

Sterilization protocol for flower buds: The flower buds were left under running tap water for 30 min, then buds were transferred to the laminar flow hood and rinsed in 70% ethanol for 1 min. The flower buds were rinsed in 10% Clorox for 10 min and finally buds were washed four times in double distilled water.

Culture media and growing conditions: The Murashige and Skoog Basel medium (MS) supplemented with 0.5 g L^{-1} Activated Charcoal (AC) was used. The media pH was adjusted to 5.8±0.1 prior to adding agar and the media were autoclaved at 121°C and 1.05 kg cm^{-2} for 15 min. Thermo-labile vitamins and growth regulators were add to the autoclaved media through membrane filters (Millex-GS 0.20 μm filter unit) under controlled conditions. Media were handed out in 10-ml into sterilized petri dishes and ten anthers were cultured in each petri dish. The petri dishes subsequently were plugged and bundled with one layer of parafilm (Pechiney Plastic Packaging, Inc, Chicago, IL.60631). The petri dishes with anthers, callus, embryos and plantlets were incubated under 16 h lights (white fluorescent light with intensity of 55 μmol m^{-2} sec^{-1}) and 8 h dark at 24±1°C.

Experiment of plant growth regulators (PGRs): The effects of BAP in combinations with 2, 4-D on the production of androgenric broccoli plants using anther culture. The experiment was

laid out in Completely Randomized Design (CRD) using 3 replicates with 5 petri dishes of each treatment. The applied plant growth regulators treatments for anther induction and embryos development were as follow: (1) 1 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D, (2) 1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D, (3) 3 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D, (4) 3 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D, (5) 5 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D and (6) 5 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D.

Experiment of sucrose media concentrations: Regarding sucrose effects on androgensis of cabbage anthers, four sucrose treatments 20, 30, 40, 50 and 60 g L^{-1} were tested using the culture medium supplemented with 1 mg L^{-1} BAP in combinations with 0.5 mg L^{-1} and 1 mg L^{-1} 2, 4-D. The experiment was laid out in Completely Randomized Design (CRD) using 3 replicates with 5 petri dishes of each treatment. The applied sucrose treatments were as follow: (1) 20 g L^{-1} sucrose+1 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D, (2) 20 g L^{-1} sucrose+1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D, (3) 30 g L^{-1} sucrose+1mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D, (4) 30 g L^{-1} sucrose+1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D, (5) 40 g L^{-1} sucrose+1 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D, (6) 40 g L^{-1} sucrose+1 mg L^{-1} BAP+1mg L^{-1} 2, 4-D, 7) 50 g L^{-1} sucrose+1 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D and (8) 50 g L^{-1} sucrose+1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D.

Assessed parameters: The following parameters were assessed from clean cultures: No. of anthers developed embryos, No. of undeveloped anthers, No. of embryos formed callus, No. of embryos formed plantlets and No. of undeveloped embryos.

Data analysis: Analysis of variance relevant to Completely Randomized Design (CRD) experiments as described by Gomez and Gomez (1984) were conducted. The treatment means were compared by the Least Significant Differences test (LSD) at 5% probability level.

RESULTS

Effects of plant growth regulators (PGRs): The development of broccoli anther was significantly influenced by the applied PGRs combinations. AS presented in Table 1 there are significant differences due to the effects of plant growth regulators combinations at all assessed traits. The applied growth regulators combinations significantly affected the anther induction and plantlets development. The culture medium with low PGRs concentrations (1 mg L^{-1} BAP+0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D) significantly increased number of anthers induced embryos comparing other applied PGRs treatments (Fig. 1a and 2c, d). The least number of developed anthers were produced when broccoli anthers were cultured on the medium supplemented with high concentrations of BAP (5 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D). On the other hand, the culture medium with lower concentration of BAP reduced significantly the number of both undeveloped anthers and dead anthers (Fig. 1b, c). Medium containing 3 and 5 mg L^{-1} BAP reduced anthers development and subsequently increased number of undeveloped and dead anthers. As presented in Fig. 1d and 2f, the highest values of embryos formed callus were obtained when the embryos were transferred to culture medium supplemented with low BAP and 2, 4-D concentrations (46.47 and 44.67 for 1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D and 1 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D, respectively). Moreover, medium with lower concentration of BAP and 2, 4-D enhanced direct differentiation of the formed embryos to plantlet (Fig. 1e and 2e-i). The highest numbers of embryos formed plantlets were 23.2 and 17.74 for culture medium 1 mg L^{-1} BAP+0.5 mg L^{-1} 2, 4-D and 1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D, respectively. Contrary,

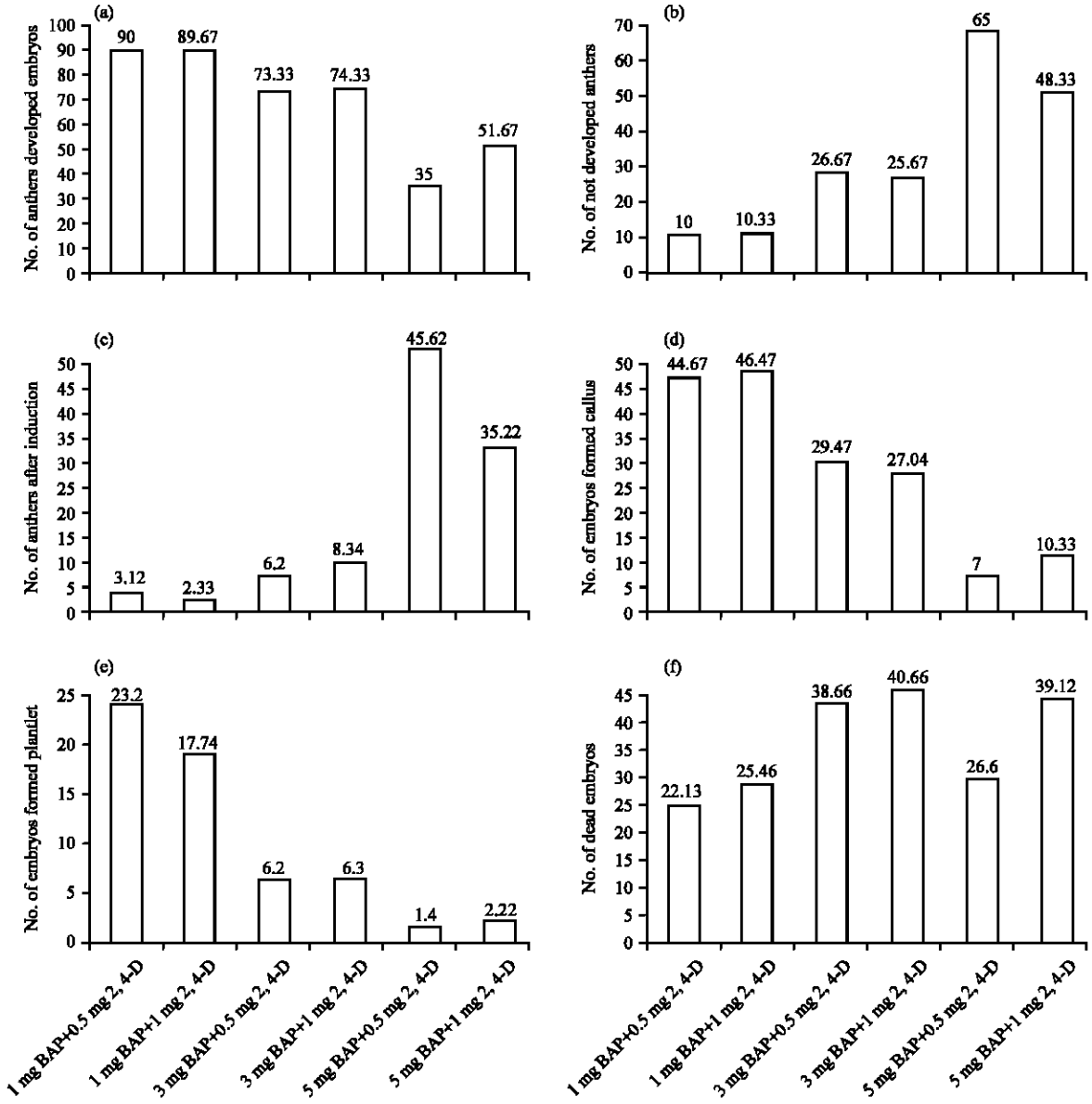

Fig. 1(a-f): Effects of plant growth regulators on androgenesis of broccoli anthers. The MS basal medium (Murashige and Skoog) supplemented with 0.5 g activated charcoal (AC) was used. Different plant growth regulators (PGRs) combinations were applied: (a) No. of anthers developed embryos ($LSD_{0.05}$ = 1.04), (b) No. of not developed anthers ($LSD_{0.05}$ = 0.261), (c) No. of dead anthers ($LSD_{0.05}$ = 0.667), (d) No. of embryos formed callus ($LSD_{0.05}$ = 0.728), (e) Embryos formed plantlets ($LSD_{0.05}$ = 0.302) and (f) No. of dead embryos ($LSD_{0.05}$ = 0.266)

increasing BAP in the culture medium form (regardless from 2, 4-D concentrations) reduced the numbers of embryos formed plantlets and increased numbers of dead embryos (Fig. 1e, f). The highest number of dead embryos were 40.66 and 39.12 for culture medium supplemented with 3 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D and 5 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D (Fig. 1f and 2g).

Table 1: Mean squares for the effects of growth regulators combinations on induction of broccoli anther

Source	df	No. of anthers developed embryos	No. of not developed anthers	No. of dead[2] developing anthers	No. of embryos formed callus	No. of embryos formed plantlet	No. of dead embryos
Rep	2	0.59	1.42*	0.26	0.71*	0.002	0.41*
PGR[1]	5	1424.23***	104.09***	10.24.79***	838.20***	227.35***	200.18***
Error	10	0.23	0.02	0.134	0.16	0.03	0.021

*,***Significant at p≤0.05 and 0.001, [1]PGR = Plant growth regulators, [2]dead anthers after the development to embryos

Table 2: Mean squares for the effects of sucrose concentrations in the culture medium on induction of broccoli anther

Source	df	No. of anthers developed embryos	No. of not developed anthers	No. of dead[2] developing anthers	No. of embryos formed callus	No. of embryos formed plantlet	No. of dead embryos
Rep	2	0.05	0.44	0.17	0.39	0.17	0.48*
Suc[1]	5	2187.21***	77.77***	1654.06***	125.58***	424.84***	238.02***
Error	10	0.28	0.02	0.18	0.12	0.06	0.06

*,***Significant at p≤0.05 and 0.001, [1]Suc = sucrose concentrations in the culture medium, [2]dead anthers after the development to embryos

Effects of sucrose concentrations: The results revealed that anther induction and development were significantly affected by sucrose concentrations in the culture medium. There are significant differences due to the effects of sucrose concentrations at all assessed traits (Table 2 and Fig. 3a-e). As presented in Table 2 the applied sucrose concentrations (20, 30, 40, 50 and 60 g L^{-1}) significantly differed in their effects on anthers induction and plantlets development. The low sucrose concentrations inspired anthers induction and embryos formation comparing to high sucrose concentrations. The highest numbers of developed anthers were 90, 90 and 89 and produced on culture medium supplemented with 20, 30 and 40 g L^{-1} of sucrose, respectively (Fig. 3a). Contrary high sucrose concentration inhibited development of broccoli anthers since the lowest number of developed anthers was observed with 60 g L^{-1} sucrose (35 anthers). High sucrose concentrations significantly increased the numbers of dead anthers after induction (Fig 3c). Regarding embryos development, low sucrose concentration significantly increased number of embryos developed plantlets with 26.94 plantlets (from 90 embryos), 24.00 plantlets (from 89 embryos) and 17.74 plantlets (from 90 embryos) and produced by 20, 40 and 30 g L^{-1}, respectively) (Fig. 3e). Thus, low sucrose concentration increased percentages of dead embryos which were 24.78% (22.13 from 90), 23.70% (21.33 from 90) and 23.42% (20.84 from 89) for 20, 40 and 30 g L^{-1}, respectively (Fig. 3f). Contrary, the numbers of embryos performed callus were significantly increased with increase sucrose concentrations. The culture medium supplemented with 60 g L^{-1} with 83.00% produced the highest percentage of embryos formed callus (29.06 from 35 developed embryos) followed by 50 g L^{-1} with 71.70% (39.25 from 54.67 developed anthers) (Fig. 3d). However, high sucrose concentrations significantly decreased percentages of dead embryos comparing to low concentration of sucrose (Fig. 3f).

DISCUSSION

High percentages of broccoli anthers were induced and developed embryos when cultured on induction medium complemented with low BAP (1 mg L^{-1}) and 2,4-D (0.5 and 1 mg L^{-1}). In cabbage anther culture, (Gorecka and Krzyzanowska, 2007) found that MS medium with low BA concentration (1 mg L^{-1}) produced the maximum percentages of embryos formed shoots (8.3%),

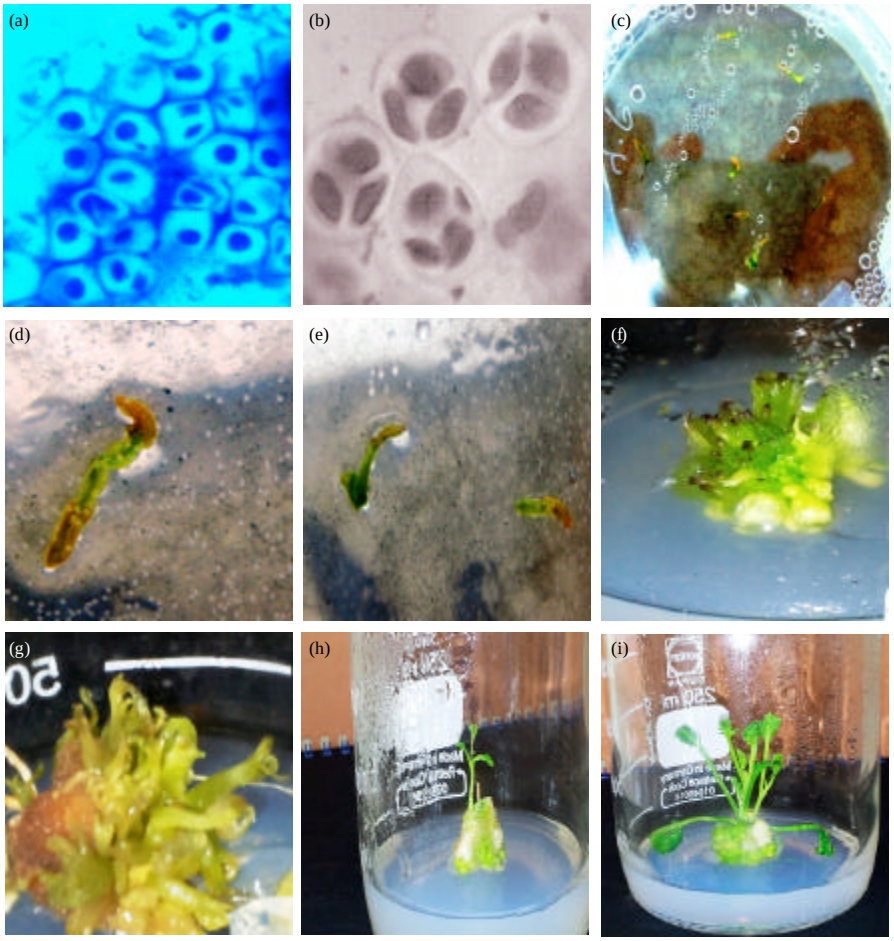

Fig. 2(a-i): Androgenesis of broccoli anthers cultured on Murashige and Skoog basal medium (MS) supplemented with 0.5 g L^{-1} activated charcoal (AC) and 3% sucrose, BAP in combinations with 2, 4-D and different sucrose concentrations: (a) Mature pollen grain, (b) The stage of microsporogenesis, (c and d) Developed anthers, (e) Anthers developed embryos, (f) Embryos formed callus, (g) Dead embryos and (h and i) Embryos formed plantlets

embryos formed callus (25%) and embryos developed into plants (25%). Hu *et al.* (1993) cultured anthers of carrot cv. 'Senkou 5 Sun' on solid MS medium containing various combinations of growth regulators. The highest rate of embryoid formation (15%) was obtained on medium containing 2, 4-D (1.0 mg L^{-1}). The low concentration of BAP and 2,4-D increased percentage of embryos formed callus and plantlet, while high BAP concentrations increased percentage of dead embryos. The results of the presented study were partially compatible to the results of Dore and Boulidard (1988) who found that embryos on a medium without growth substances transformed into single complete plants, whereas from embryos placed on a medium with 0.1 mg L^{-1} BA emerged shoots. George *et al.* (2008) found that BAP was most effective in enhancing shoot multiplication and elongation. Moreover, they found that BAP enhance first the differentiation of cell into shoot and subsequently the formation of shoots. Ravanfar *et al.* (2009) reported that high concentration

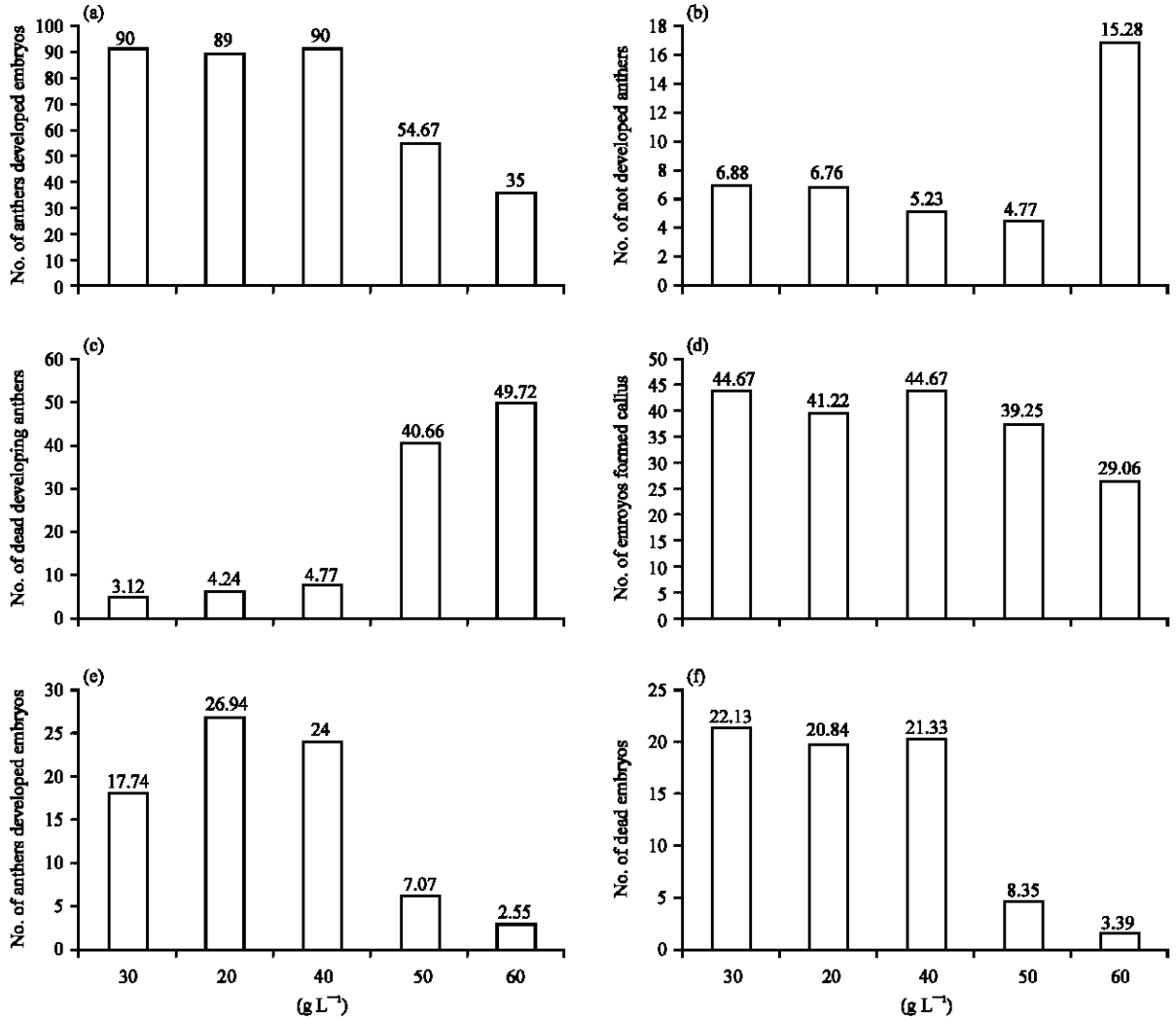

Fig. 3(a-f): Effects of sucrose concentrations (20, 30, 40, 50 and 60 g L^{-1}) on androgenesis of broccoli anthers. The MS basal medium (Murashige and Skoog) supplemented with 0.5 g activated charcoal (AC) was used. Different plant growth regulators (PGRs) combinations were applied: (a) No. of anthers developed embryos ($LSD_{0.05}$ = 1.053), (b) No. of not developed anthers ($LSD_{0.05}$ = 0.593), (c) No. of dead anthers ($LSD_{0.05}$ = 0.850), (d) No. of embryos formed callus ($LSD_{0.05}$ = 0.686), (e) Embryos formed plantlets ($LSD_{0.05}$ = 0.509) and (f) No. dead embryos ($LSD_{0.05}$ = 0.489)

of BAP (5 mg L^{-1}) decreased the mean number of shoots formed per explant due to the toxicity to the shoot growth. The results of the five sucrose concentrations showed that high percentages of anthers induction and embryos development were observed when broccoli anthers were cultured on medium supplemented with low sucrose concentrations. Low sucrose concentration significantly increased the No. of embryos produced plantlets, while increased the No. of dead embryos. The results were partially agreed with that reported by Roulund *et al.* (1990). The authors studied the effects of sugar concentrations and types on another culture of head cabbage. They reported that a higher average response was on the sucrose containing media (3.4 embryos/100 anthers) compared to the media with maltose (1.6 embryos/100 anthers). Also, Zhang *et al.* (2006) reported

that the B5 media supplemented with 2 mg L^{-1} 2, 4-D, 2 mg L^{-1} kinetin and 60 g L^{-1} sucrose was the optimal medium for embryoid induction of cabbage anthers. The induction and stimulation of broccoli anther[s] were significantly restricted by increasing the sucrose concentrations. High sucrose concentrations increased percentages of embryos performed callus, while decreased percentages of dead embryos comparing to low concentration of sucrose. However, Gorecka and Krzyzanowska (2007) found that B5 medium without amino-acids and hormones and containing 20 g L^{-1} sucrose revealed the lowest percentages of embryos formed shoots (0%), embryos formed callus (0%) and embryos developed into plants (0%). Krzyzanowska *et al.* (2006) reported that MS with 20 g L^{-1} sucrose, 1 mg L^{-1} BA and 0.001 mg L^{-1} NAA showed the highest percentages of embryos producing shoots (15.1%).

CONCLUSION

As conclusion, BAP in combinations with 2, 4-D can be successfully used to perform embryos and/or callus from the callus induction and embryos development either to callus or plants. The low concentrations of sucrose revealed the superlative results of percentages of induced anthers and embryos developed into callus and plantlets.

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